

***Anchusa italica* extract: phytochemical and neuroprotective evaluation on global cerebral ischemia and reperfusion**

Akram Torki¹, Valiollah Khalaji-Pirbalouty¹, Zahra Lorigooini², Mahmoud Rafieian-Kopaei^{2,*}, Arezo Sadeghimanesh¹, Zahra Rabiei²

¹Department of Biology, Faculty of Science, Shahrekord University, Shahrekord, Iran, ²Medicinal Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

Stroke is the third leading cause of mortality and disability in industrial countries. Treatment with herbs with antioxidant properties has been reported to be an alternative to the conventional treatments. This study was conducted to investigate the effect of *Anchusa italica* extract on hippocampal injury induced by transient global cerebral ischemia and reperfusion in the rat. To do so, 50 rats were randomly assigned to five groups; control, sham, ischemia, and 50 or 100 mg/kg of *Anchusa italica* treated animals. Ischemia was induced by occlusion of carotid artery for 30 minutes. Afterward, behavioral tests and biochemical analyses were conducted. Induction of ischemia/reperfusion caused a decline in learning and passive avoidance memory in rats. Moreover, *Anchusa italica* caused an increase in learning and improved the passive avoidance memory. Induction of ischemia/reperfusion caused a decrease in the antioxidant capacity of the brain and serum as well as an increase in the malondialdehyde of the brain and serum. *Anchusa italica* led to an increase in the antioxidant capacity of the brain and serum and decrease in the malondialdehyde of the brain and serum. Overall, because of its protective effects on spatial memory, passive avoidance learning, antioxidant capacity, and lipid peroxidation during ischemia/reperfusion, *Anchusa italica* might be beneficial in ischemic patients.

Keywords: Boraginaceae/phytochemistry. Brain ischemia. Spatial memory/drug effects. Malondialdehyde/pharmacology. Neuroprotection/drug effects. Lipidic peroxidation/drug effects. Antioxidant capacity. Passive avoidance memory.

INTRODUCTION

Cerebral ischemia is one of the most debilitating brain injuries and the most important cause of mortality worldwide particularly in people over 65 years. Different factors are effective in inducing cerebral ischemia including cardiac arrest, atherosclerosis, thromboembolic events, vasoconstriction, congenital heart disorders, low blood pressure, and some types of tumors (Thom *et al.*, 2006; Bernard *et al.*, 2002; Pourheydar *et al.*, 2014). Neurological events due to cerebral ischemia include sensory, movement, and visual disorders as well as aphasia, agnosia, amnesia, and impaired spatial learning and memory (Andrews *et al.*, 2008; Bokura, Robinson, 1997).

Hippocampus, as an organ involved in the formation of new memory and processing spatial information, is the most sensitive area of the brain during ischemia, trauma, and degenerative diseases (Zhang, Piantadosi, 1994; Deckert, Jorgensen, 1988). During cerebral ischemia, generation of free radicals including reactive oxygen (ROS) and nitrogen species (RNS) brings damage to lipids, proteins, and desoxyribonucleic acid, resulting in neuronal death. Free radicals play an important role in breaking the blood-brain barrier and inducing cerebral edema (Wolff, Dean, 1987). Indeed, oxidative stress extensively influences cell membrane system particularly membrane bilayer phospholipids. Therefore, it can be argued that lipids peroxidation is oxidative damage to lipids with carbon double bonds (White *et al.*, 2000; Oruc *et al.*, 2004). The final product of lipids peroxidation is malondialdehyde (MDA), which is a mutagenic, carcinogenic, and highly reactive compound (Janero, 1990).

*Correspondence: M. Rafieian-kopaei. Head of Medical Plants Research Center. Editor-in-Chief of JSKUMS and J HerbMed Pharmacol, Shahrekord University of Medical Sciences, Shahrekord, Iran. Tel: +98 381 334 6692 Mob: +989131811842 Fax: +98 381 3330709. E-mail: rafieian@skums.ac.ir; rafieian@yahoo.com

Use of antioxidants is an approach to reduce adverse effects of cerebral ischemia (Dekanski *et al.*, 2011). Recently, many synthetic antioxidants have been found to be carcinogenic and toxic and therefore some efforts have been made to identify nature-based antioxidants (Zhang *et al.*, 2006; Gulluce *et al.*, 2007).

Anchusa italica, locally called as “Gav zaban” in Persian, is one of the important genus of family Boraginaceae. Five species of genus *Anchusa* have been found in Iran (Mozaffarian, 1996; Kazemi, 2013). *A. italica* has been demonstrated to contain alkaloids, tannins, saponins, triterpenes, and polyphenols such as anthocyanins and flavonoids (Barbakadze *et al.*, 2010). In traditional medicine, *A. italica* extract is used as a stimulant, tonic, palliative in biliary diseases, diuretic, sedative, antiarthritic, anti-asthma, and laxative. It is also used to reduce fever and cough (Khare, 2008; Kebriaee-Zadeh, 2010). *A. italica* has been reported to have antioxidant, anticancer, and antiviral effects as well as certain effects on the central nervous system and endogenous glands (Al-Snafi, 2014). In the light of *A. italica* antioxidant effects, this study was conducted to investigate neuroprotective effects of hydroalcoholic extract from *A. italica* flowers on hippocampal injury induced by transient global cerebral ischemia and reperfusion in the rat.

MATERIAL AND METHODS

Plant material and extraction

A. italica flowers were gathered and botanically confirmed by a botanist and deposited as specimens in Herbarium of Medical Plants Research Center in Shahrekord University of Medical Sciences, Shahrekord, Iran (SKUMS-504). The dried and pulverized sample was extracted using ethanol 70%. The resulting extract was filtered and concentrated using a rotary evaporator. To conduct final drying, the concentrated extract was incubated at 37 °C.

Determining antioxidant capacity of the extract and measuring total phenol and flavonoid content

After the stock of extract and DPPH was prepared, the sample was incubated in the dark for 15 minutes and the samples' absorbance was read at 517 nm wavelength. Methanol was used as blank and methanol plus DPPH was considered a control. After calculating the inhibition rate of free radicals by the extract, the antioxidant activity was reported as IC₅₀ (McDonald *et al.*, 2001).

The total phenol of *A. italica* was measured by Folin-Ciocalteu reagent and aluminum chloride colorimetric method (Chang *et al.*, 2002; Asgari *et al.*, 2012). The experiments were conducted in triplicate and the total phenol and flavonoid content of the extract was measured and reported in mg/g dried extract.

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animal and ethical guidelines of IR. SKUMS Bioethics Committee (Rec.2015.141). In this study, 50 male adult Wistar rats weighing 250-300 g were used. The rats were kept at 21±2 °C temperature, under 12-h dark/12-h light cycle and had free access to the same food and water. The rats were assigned to five groups as follows: 1) Control group that underwent no surgery and received distilled water alone, 2) Sham group that underwent surgery without drug administration and occlusion of carotid arteries. This group also received distilled water alone, 3) Ischemia group that underwent ischemia without drug administration received distilled water alone, and Groups 4) and 5) received *A. italica* extract (50 and 100 mg/kg, respectively) with induction of ischemia. The extract was administered intraperitoneally (IP) (Mrudula, 2014; Ghahremanitamadon *et al.*, 2014). In intraperitoneal injection, the rate of absorption of the drug was higher than oral administration. Drugs were administered after the induction of ischemia model.

Induction of ischemia

The global cerebral ischemia is created with severe blood reduction or occlusion to the brain. The ischemia model in this study was performed with general carotid arteries occlusion of bilateral vessels (Kawai *et al.*, 1992; Lipton, 1999). After induction of anesthesia using 400 mg/kg of chloral hydrate, the neck anterior lateral region underwent surgery after carotid sheath was determined, and common carotid arteries were carefully identified from vagosympathetic nerve and separated. Then, carotid arteries were occluded for 30 minutes and then reperfusion was performed. Behavioral tests were performed one week after induction of ischemia. After performing the behavioral tests and inducing anesthesia for the second time, the blood samples were taken from the hearts. Finally, hippocampus, cortex, and sub-cortex that were already taken out from the brain were separated on the ice and analyzed biochemically. The blood samples were centrifuged and then the serum isolated and used for biochemical analyses.

Morris water maze test and shuttle box test

In this test, each rat was given an opportunity of 60 seconds to find the platform. If the rat could not find the platform, the researcher would guide it to the platform. Between each pair of the trials, the rats could rest for 30 seconds to examine the surrounding. Between two blocks, the rats were taken out from water for 10 minutes and left to rest in the cage. Each rat was trained four times per day for four days and the test was repeated on day 5 without the platform that was considered “probe day” (Dominique *et al.*, 1998).

The shuttle box test lasted for four days for each rat. On days 1 and 2 of the test, each rat was placed inside the instrument to adapt to it. On day 3, the acquisition was examined by putting them separately into a bright room. After a 2-minute period of acculturation, the guillotine door was opened and after the rat entered the dark room it was closed and an electrical shock (1 mA, 1 second, once) was applied on the rat such that it just paddled. In this test, the primary latency to enter the dark room was recorded on the day 3 and the latency during passing was recorded on the day 4 (Khalili, Roghani, Ekhlasi, 2010).

Measuring antioxidant capacity of the serum and the brain

For measuring antioxidant capacity of the serum and the brain, three solutions were used: a buffer (1.55 mL sodium acetate, 8 mL concentrated acetic acid reaching 500 mL by adding distilled water), a ferrous chloride solution (270 mg FeCl₃ (6 H₂O) reaching 50 mL by adding distilled water), and triazine solution (47 mg triazine dissolved in 40 mL of 40 mM HCl). The labor solution was prepared by addition of 10 mL of the first solution, 1 mL of the second solution, and 1 mL of the third solution. Next, 25 μ L of the serum sample and 25 μ L of the homogenized brain sample were added to 1.5 mL of the labor solution and kept at 37 °C. Optical absorbance was read at 593 nm wavelength (Benzie, Strain, 1999).

Measuring serum and brain MDA

To measure the amount of the serum MDA, 0.5 g of thiobarbituric acid was mixed with 80 mL acetic acid 20% and then the pH was set at 3.5 using NaOH and its volume reached 100 mL by adding acetic acid 20%. After that, 100 μ L of the serum sample was mixed with 100 μ L of SDS 8.1% and 2.5 mL of the labor solution. The samples were placed in a bain-marie of boiling water for 60 minutes and then cooled and centrifuged at 4000 rpm.

The optical absorbance of the supernatant was read at 523 nm wavelength.

To measure the brain MDA, 1 g of the brain tissue was cooled with KCL 2.5% and homogenized at 10% (weight-volume) proportion, and then incubated at 37 \pm 1 °C for 60 minutes in a metabolic shaker. Then, 1 mL of trichloroacetic acid 5% and 1 mL of thiobarbituric acid 67% were added to it and mixed well after each step. The content of each vial was transferred to the centrifugation tube and centrifuged at 2000 rpm for 15 minutes. Then, the supernatant was transferred to another tube and placed in a bain-marie. After 10 minutes, the tubes were cooled, and the optical absorbance read at 535 nm wavelength (Karatas, Karatepe, Baysar, 2002).

Data analysis

The data were expressed as mean \pm SEM and analyzed by one-way ANOVA and Tukey's post-hoc tests in GraphPad Prism Software version 5. The level of significance was considered at $P < 0.05$.

RESULTS

Total phenol and flavonoid content

The total phenol and flavonoid contents of *A. italica* extract were derived 62.2 \pm 0.3 and 47.06 \pm 0.4 mg/g of the dried extract, respectively. Scavenging capacity of radicals and antioxidant activity of *A. italica* extract (IC₅₀) was derived about 90.52 \pm 0.3 μ g/mL.

The effect of *A. italica* extract on learning and spatial memory in Morris Water Maze test

The findings of Morris Water Maze test demonstrated that latency to find the platform was the highest in the ischemic group and lowest in the control group. More clearly, ischemia caused learning and memory deficit. In the extract-treated groups, memory and learning improved. Intraperitoneal administration of 50 and 100 mg/kg of the extract caused a significant decrease in the latency to find the platform compared to the ischemia group ($p < 0.001$). There was no significant difference in the latency among the control, sham, and the extract (50 and 100 mg/kg) groups ($p > 0.05$). Also, there was no significant difference between the two extract (50 and 100 mg/kg) treated groups ($p > 0.05$) (Figure 1).

Latency to find the platform was highest in the ischemic group and lowest in the control group. Intraperitoneal administration of 50 and 100 mg/kg of the

extract caused a significant decrease in the latency to find the platform compared to the ischemic group ($p < 0.001$). Similarly, there was no significant difference in the latency time among the control, sham, and the extract 100 mg/kg groups ($p > 0.05$). There was a significant difference between two extract-treated (50 and 100 mg/kg) groups ($p < 0.001$) (Figure 1).

Duration of swimming in target zone on the probe day was longest in the control group and shortest in the ischemia group. Intraperitoneal administration of 50 and 100 mg/kg of the extract caused a significant increase in the duration of swimming in target zone compared to the ischemia group ($p < 0.001$). However, there was no significant difference in the duration of swimming in target zone among the control, sham, and the extract (50 and 100 mg/kg) groups ($p > 0.05$). Also, there was no significant difference between the two extract (50 and 100 mg/kg) treated groups ($p > 0.05$) (Figure 1).

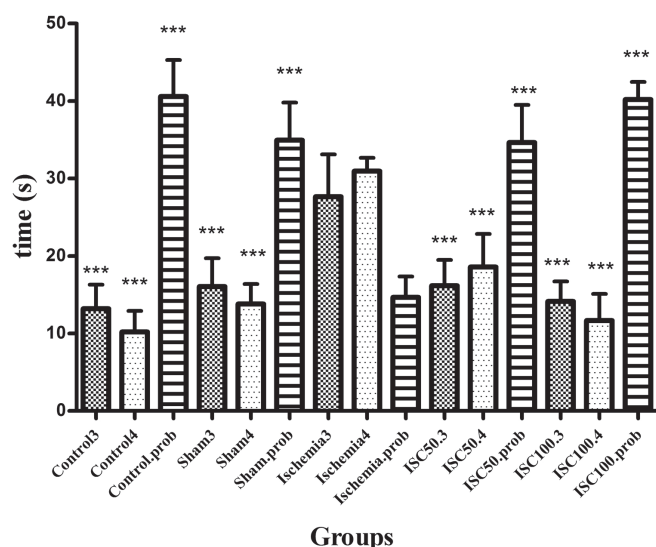


FIGURE 1 - The effect of *A. italica* extract on learning and spatial memory in Morris water maze test on hippocampal injury induced by transient global cerebral ischemia and reperfusion. time: latency to find the platform on day 3, 4 and duration of swimming in target zone on the probe day. ISC 100 and ISC 50: treated with 100 and 50 mg/kg of *Anchusa italica* extract, respectively. (***: significant difference between the all groups with ischemia group, $p < 0.001$). The number of samples, $n = 7$.

The effect of *A. italica* extract on passive avoidance memory in Shuttle Box test

The findings demonstrated that the time difference between the primary and secondary latency was highly marked in the control group. This difference in the ischemia group was negligible. In the extract-treated

groups, the time difference decreased with an increase in the extract dose. The secondary latency was highest in the control group and lowest in the ischemia group. Intraperitoneal administrations of 50 and 100 mg/kg caused a significant increase in the secondary latency compared to the ischemia group ($p < 0.001$). There was no significant difference in the secondary latency among the control, sham, and the extract 100 mg/kg groups ($p > 0.05$). Moreover, there was a significant difference between the two extract (50 and 100 mg/kg) treated groups ($p < 0.05$) (Figure 2). In this test, the latency during passing was considered as the secondary latency time.

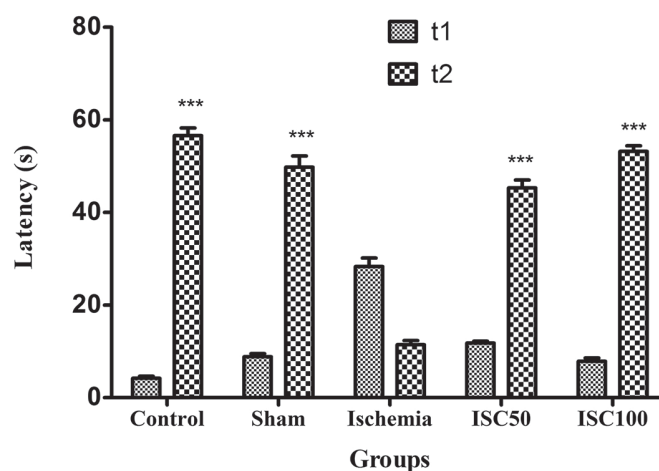


FIGURE 2 - The effect of *A. italica* extract on passive avoidance memory in shuttle box test on hippocampal injury induced by transient global cerebral ischemia and reperfusion. t1: the primary latency to enter the dark room and t2: the latency during passing. ISC 100 and ISC 50: treated with 100 and 50 mg/kg of *Anchusa italica* extract, respectively. (***: significant difference between the all groups with ischemia group, $P < 0.001$). The number of samples, $n = 7$.

The effect of *A. italica* extract on antioxidant capacity of serum and brain

The antioxidant capacity of the serum was significantly higher in the rats treated with 50 and 100 mg/kg of the *A. italica* extract than in the ischemic group ($p < 0.01$ and $p < 0.001$, respectively). Moreover, the antioxidant capacity of the brain was significantly higher in the rats treated with 50 and 100 mg/kg of the *A. italica* extract than in the ischemia group ($p < 0.05$ and $p < 0.001$). The antioxidant capacity of the serum and brain was not significantly different between the control, sham and the extract 100 mg/kg groups ($p > 0.05$). There was a significant difference between the two treated groups ($p < 0.001$) (Figure 3).

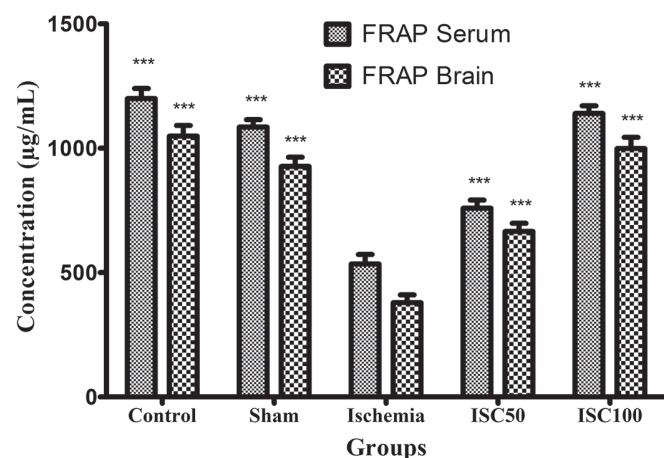


FIGURE 3 - The effect of *A. italica* extract on antioxidant capacity of serum and brain on hippocampal injury induced by transient global cerebral ischemia and reperfusion. ISC 100 and ISC 50: treated with 100 and 50 mg/kg of *A. italica* extract, respectively. (***: significant difference between the all groups with ischemia group, $P < 0.001$). The number of samples, $n = 7$.

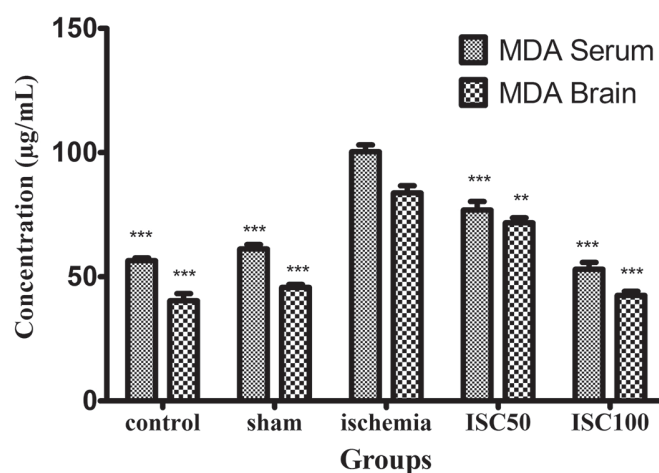


FIGURE 4 - The effect of *A. italica* extract on MDA of serum and brain on hippocampal injury induced by transient global cerebral ischemia and reperfusion. ISC 100 and ISC 50: treated with 100 and 50 mg/kg of *A. italica* extract, respectively. (***: significant difference between the all groups with ischemia group, $P < 0.001$, $P < 0.01$). The number of samples, $n = 7$.

The effect of *A. italica* extract on MDA of serum and brain

The serum MDA level was significantly lower in the rats treated with 50 and 100 mg/kg of the *A. italica* extract than in the ischemia group ($p < 0.001$). Moreover, the brain MDA level was significantly lower in the rats treated with 50 and 100 mg/kg of the *A. italica* extract than in the ischemia group ($p < 0.05$ and $p < 0.001$). The serum and brain MDA levels were not significantly different among the control, sham and the extract (100 mg/kg) groups ($p > 0.05$); however, there was a significant difference between the two extract (50 and 100 mg/kg) treated groups ($p < 0.001$) (Figure 4).

DISCUSSION

The importance of oxidative stress in cerebral ischemia

Regarding the significance of stroke as one of the important health issues worldwide, many studies have been conducted to investigate the factors involved in pathogenesis as well as prevention and treatment of stroke. The positive effects of herbal drugs in mitigating adverse effects of ischemia/reperfusion have already been demonstrated. Medicinal plants and some of their active compounds have been demonstrated to minimize brain lesion volume, cerebral edema, neuronal injury, and

ischemia-induced consequences. The action mechanisms of protective effects of medicinal plants include a decrease in oxidative and nitrative stress, decrease in lipids peroxidation, decrease in the activities of microglia and astrocytes, inhibition of apoptotic proteins expression, increase in expression of mitochondrial genes, prevention of fragmentation and oxidative damage to DNA, increase the expression of antiapoptotic proteins, and decrease in expression of inflammation mediators (Rabiei, Bigdeli, Lorigooini, 2015).

Cerebral ischemia causes initiation of signaling cascade that leads to the production of free radicals and oxidative stress depending on the severity and duration of the complication. Oxidative stress is an important factor for cerebral dysfunction in neurodegenerative diseases such as ischemia (Lipton, 1999). Oxidative stress due to ischemia can enhance the production of free radicals and lipid peroxidation. Lipid peroxidation has been reported to increase in the early stages of ischemia (El Kossi, Zakhary, 2000; Ozkul *et al.*, 2007; Aygul *et al.*, 2006). Oxidative stress not only causes an increase in production of free radicals but also reduces the cellular antioxidant mechanisms (Açikgöz *et al.*, 1998).

Antioxidants have attracted much attention in the development of neuroprotective agents to treat stroke. The physiological role of antioxidants is seen in scavenging free radicals (Köksal, Gülçin, 2008). Plant antioxidants have been recommended as potential agents to prevent cerebral damage or to reduce and treat cerebral damage

due to ischemia/reperfusion. Therefore, studying the effects of antioxidants and free radical-trapping agents is important since they are fundamentally protective agents against various brain damage such as cerebral damage due to ischemia/reperfusion (Baradaran, Nasri, Rafieian-Kopaei, 2013).

The antioxidant effect of *A. italica* extract

In the current study, the brain and serum antioxidant capacities in the ischemia group decreased compared to the control group. Moreover, in the *A. italica*-treated groups, the brain and serum antioxidant capacities increased compared to the ischemia group, suggesting an improvement of these variables after administration with the extract.

This study demonstrated that the serum and brain MDA was higher in the ischemia group compared to the control group. Besides, in the extract-treated groups, the serum and brain MDA decreased compared to the ischemia group. It seems that this finding is due to the protective effect of *A. italica* extract components in reducing lipid peroxidation. The role of plant flavonoids has been demonstrated in reducing lipid peroxidation. Akhlaghi and Bandy (2009) reported that the use of flavonoids caused a decrease in production of MDA and free radicals such as superoxide, pyroxyl, and peroxyxynitrite during ischemia. Pataki *et al.* (2002) studied the effect of flavonoids on ischemia and found that these compounds decrease the production of ROS during ischemia-reperfusion.

A. italica extract was reported to have a wide spectrum of pharmacological and therapeutic effects including antioxidant and anti-inflammatory activities (Al-Snafi, 2014). Another study has indicated that borage leaf extract is able to eliminate ROS and DPPH radicals (Wettasinghe, Shahidi, 2000). Elsewhere, the antioxidative activity decreased with a decrease in phenolic compounds (Oms-Oliu *et al.*, 2008). In the current study, *A. italica* extract displayed acceptable antioxidant activity to inhibit DPPH radicals. Therefore, this ability appears to be related to the antioxidant property of phenolic and flavonoid compounds present in *A. italica* leaf extract.

In this study, *A. italica* extract was found to contain high amounts of phenolic and flavonoid contents. The effect of *A. italica* extract in controlling ischemia seems to be because of the presence of these compounds.

Antioxidants activities are due to secondary metabolites such as polyphenols (Baradaran, Nasri, Rafieian-Kopaei, 2013). Antioxidant activities of the flavonoids are likely to be much more important. Flavonoids exert antioxidant activity against free

radicals and have been known as important factors for inhibiting lipids peroxidation (Middleton, Kandaswami, Theoharides, 2000; Rafieian-Kopaei, Baradaran, Rafieian, 2013).

Zhang *et al.* (2013) studied flavonoids effects on ischemia/reperfusion-induced damage and found that these compounds prevented the fragmentation of DNA and increased expression of Bcl-2. In addition, they caused a decrease in expression of p53, Apaf1, fas, fasL, Bax, Bid, cytochrome C, and caspase 3, 8, and 9. The findings of this study revealed that flavonoid compounds cause a decrease in expression of NF-KB, iNos, MMP9, Cox-2, TNF- α , IL-1 β , IL-4, and IL-6. Therefore, it seems that flavonoids have potential to treat ischemia because of their antioxidant, antiapoptotic, and anti-inflammatory properties.

Hi *et al.* (2012) demonstrated that saponins could exert protective effects against cerebral ischemia through decreasing MDA, lactate dehydrogenase, xanthine oxidase, caspase 3, and Bax gene and increasing SOD and glutathione peroxidase. A study showed that saponins could also exert antiapoptotic effects in ischemia through inhibiting P13K pathway (Chen *et al.*, 2011). *A. italica* has been shown to contain saponins (Al-Snafi, 2014); therefore, it seems that protective effect of this plant on cerebral ischemia is due to the presence of these compounds.

Morris Water Maze test revealed that in the ischemia group, latency to find the platform in target zone was much longer than that of the control group. This duration decreased in the extract-treated groups compared to the ischemia group. Duration of swimming in target zone on the probe day in the ischemia group was much shorter than that of the control group and increased in the extract-treated groups compared to the ischemia group. This finding can represent the improvement of spatial memory and learn to recall the platform location in *A. italica* extract-treated groups. Pu *et al.* (2007) investigated the effects of four flavonoids in cerebral ischemia model. These flavonoid compounds caused improvement of spatial memory deficit and neuronal death induced by cerebral ischemia (Pu *et al.*, 2007). Shang *et al.* (2006) induced ischemia in rats to study the effects of flavonoids in treating this disorder and found that these compounds led to an improvement in learning and spatial memory of these rats.

Shuttle Box test demonstrated that the difference between t_1 and t_2 in the ischemia group was slight, which indicates avoidance memory deficit is due to ischemia. Moreover, this difference in *A. italica*-treated groups was much more marked than that of the ischemia group, which indicates the optimal effect of this extract on passive avoidance memory.

CONCLUSION

The findings of this study showed that inducing ischemia-reperfusion in Wistar rats caused spatial and passive avoidance memory deficit in these rats decreased the antioxidant capacity of the serum and the brain, and increased the brain and serum MDA. Intraperitoneal administration of *A. italica* hydroalcoholic extract for 14 days could significantly improve ischemia/reperfusion-induced injury on memory behaviors and the brain and serum biochemical changes. In the light of these findings, it seems that *A. italica* hydroalcoholic extract caused improvement of ischemia-induced injuries through scavenging free radicals derived from ischemia/reperfusion process owing to containing abundant antioxidant compounds (phenolic and flavonoid compounds and saponins).

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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